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Role of Insulin-like growth factor-2 in colorectal cancer.（大腸癌における
Insulin-like growth factor-2の役割）　河本　和幸

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Role of Insulin-like growth factor-2 in colorectal cancer.
（大腸癌におけるInsulin-like growth factor-2の役割）

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Possible Paracrine Mechanism of Insulin-Like Growth Factor-2 in the Development of Liver Metastasis from Colorectal Cancer.

Running title: Role of IGF-2 in liver metastasis

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Precis : Hepatocyte-derived IGF-2 stimulates cancer cell proliferation by paracrine mechanism and may play an important role in colorectal cancer patients with liver metastasis.

(1) Text pages : 21

(2) Tables : 3

(3) Illustrations : 9

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Abstract

BACKGROUND. IGF-2 is considered one of the autocrine growth factors in colorectal cancer. In addition, it is well-known that IGF-2 is produced in the liver. However, the role of IGF-2 in liver metastasis is not yet clearly understood.

METHODS. Immunohistochemical staining of IGF-2 and IGF-1R was performed on tissue samples of liver metastase from 30 colorectal cancer patients. In situ hybridization of IGF-2 was also subjected to the same tissue samples. Furthermore, PCNA was immunohistochemically stained for use as an indicator of the proliferative activity of cancer cells.

RESULTS. Invasive margins of liver metastasis were highly stained by both of IGF-2(70%) and IGF-1R staining(83%). Overexpression of IGF-2 protein and mRNA was observed in the normal liver adjacent to the tumor. The PCNA LIs for the IGF-2 positive groups were significantly higher than those for the IGF-2 negative group($p<0.0001$). In addition, the PCNA LIs for the IGF-1R positive groups were also significantly higher than those for the IGF-1R negative group($p=0.0002$).

CONCLUSION. Our findings suggest that hepatocyte-derived IGF-2 stimulates cancer cell proliferation by paracrine mechanism and plays

an important role in tumor progression in colorectal cancer patients with liver metastasis.

key words : Insulin-like growth factor-2, Insulin-like growth factor-1 receptor, colorectal cancer, liver metastasis, paracrine mechanism, proliferating cell nuclear antigen.

Introduction

The liver is the most common site for visceral metastasis, and liver metastasis often leads to poor prognosis in patients with colorectal cancer. To produce visceral metastasis, cancer cells must clear all steps, that is progressive growth at the primary organ, angiogenesis, invasion to microvessels, cell detachment, survival in the circulation, adhesion to microvessels and growth at the target organ[1]. A variety of factors are involved in this process, such as angiogenic factors, matrix metalloproteinases, cellular adhesion molecules, growth factors and so on. In order to overcome liver metastasis, it is necessary to identify the role of each of these factors.

In 1889, Paget[2] proposed the "seed and soil theory", which held that "The organ microenvironment (soil) can influence the growth of particular tumor cells (seed)". It seems that if the proliferation of cancer cells is stimulated by hepatocyte-derived growth factors, the cancer cells are suitable to survive in the liver. Some growth factors have been identified as such. For example, Radinsky[3] reported that Transforming growth factor(TGF)-α was such a growth factor, and Li Long et al[4] described the role of insulin-like growth factor(IGF)-1 and IGF-1 receptor(IGF-1R).

Previously we reported that the expression of IGF-2 in primary colorectal cancer was related to tumor progression and patient survival, with the implication that IGF-2 could be one of the autocrine growth factors in colorectal cancer[5]. IGF-2 is a small, single-chain polypeptide (MW:7471), which plays an important role in fetal growth. After birth, IGF-2 levels decrease, and only a few tissues, for example liver and nerves, express IGF-2 in the adult[6-9]. Overexpression of IGF-2, however, has been reported in many types of tumors, including breast cancer, hepatoma, smooth muscle tumors, liposarcoma, colorectal cancer and others[10-14]. Furthermore, recent reports have suggested that IGF-2 acts as an autocrine growth factor by binding to IGF-1R in several human colorectal cancer cell lines[15-17].

From the fact that the liver produces IGF-2, we hypothesized that hepatocyte-derived IGF-2 acted on colorectal cancer cells by a paracrine mechanism in the liver and promoted cancer cell proliferation and tumor progression. In the present study, IGF-2 and IGF-1R expression was studied by means of immunohistochemical staining of paraffin-embedded specimens of liver metastasis in colorectal cancer patients. In addition, the messenger RNA(mRNA) expression of IGF-2 was examined by using in situ hybridization. The relationship between proliferating

cell nuclear antigen(PCNA) and proliferative activity has recently been confirmed[18,19], so that immunohistochemical staining of PCNA was used in this study to provide an indicator of the proliferative activity of cancer cells.

Materials and Methods

1) Immunohistochemical analysis of IGF-2 and IGF-1R. The labelled streptavidin biotin(LSAB) method(Dako LSAB kit; Dako Japan Co., Kyoto, Japan) was used for immunohistochemical staining of IGF-2 and IGF-1R in paraffin-embedded specimens obtained from 30 patients who underwent surgical treatment for liver metastasis from colorectal cancer between April 1, 1987 and March 31, 1996, in the Department of Surgery and Surgical Basic Science, Graduate School of Medicine, Kyoto University, Kyoto, Japan. There were 16 males and 14 females with a mean age of 62.6 years(ranging from 45 to 78). There were 16 patients with synchronous hepatic metastasis and 14 patients with metachronous hepatic metastasis(Table1). One block with both cancer and normal liver tissue was selected from each case, following examination of hematoxylin and eosin-stained slides of the surgical specimens. Sections were cut to 5 μm thickness, and mounted on

silanized slides. The sections were then deparaffinized in xylene and rehydrated. After endogenous peroxidase activity was quenched with 3% hydrogen peroxide, nonspecific binding was blocked by preincubation with normal bovine serum. The slides were then incubated overnight at 4℃ with anti-IGF-2 antibody(No.05-166; UBI Co., New York, N.Y., U.S.A.) at a final concentration of 10 μg / ml in phosphate buffered saline(PBS), pH 7.4. Next, the slides were washed with PBS, incubated with biotynylated goat anti-rabbit secondary antibody for 10 minutes, again washed with PBS, and incubated with peroxidase-labelled streptavidin for 10 minutes. Finally, 3-amino-9-ethylcarbazol(AEC) was used as a chromagen, and the slides were counterstained with hematoxylin. IGF-1R staining was examined in serial sections of 30 tissues from the patients examined for IGF-2 staining. Immunohistochemical staining of IGF-1R was performed by the same method as described for IGF-2 staining except for the use of anti-IGF-1R antibody(No.GR-11; Calbiochem Co., Cambridge, U.K.) as the primary antibody and ENVISION Polymer Reagent(No.K1490; Dako Japan Co., Kyoto, Japan) as the secondary antibody. All slides were observed microscopically at 400× magnification, and the ratio(%) of IGF-2 positive cells to all cells examined(about 400 cells) was calculated

at 4 sites: the center of the tumor, the invasive margin of the tumor, the normal liver adjacent to the tumor and the normal liver at least 1cm distant from the tumor(Fig.1). The ratio(%) of IGF-1R was calculated at the invasive margin of the tumor. Scoring was performed independently by two observers(K.K., H.O.) without knowledge of patient status and the mean of their scores was used for the following analyses. If the ratio of positive cells was greater than 10%, the tissue was designated as "positive", while tissues with a ratio of less than 10% were classified as "negative". Furthermore, a ratio of greater than 50% was labeled "strongly positive". The cut-off point of 10% was chosen on the basis of our previous study, which showed that this delineation point differentiated cancer cells from normal mucosa with the smallest margin of error.

2) In situ hybridization of IGF-2 Serial paraffin sections of 30 tissues from the patients examined for IGF-2 and IGF-1R staining were used for in situ hybridization of IGF-2. In situ hybridization was performed according to the protocol of the "DNA Probe Hybridization/Detection System-In Situ Ultra-Sensitive Kit" (No. IHD-0052, MBI Co. San Francisco, U.S.A.). A biotinylated IGF-2 probe(No. 2328-0101, GSI Co. South San Francisco, U.S.A.) was used for

hybridization. To detect the biotinylated probe hybridized to the target sequence, an anti-biotin antibody was applied first, followed by the addition of immunoglobulin to re-introduce biotin into the system. The single-component BCIP/NBT solution was added as a chromagen. Finally, slides were mounted in a permanent mounting medium. An intense dark-brown signal was observed at the specific site of the hybridized probe.

3) Immunohistochemical analysis of PCNA. PCNA staining was examined in serial sections of 30 tissues from the patients examined for IGF-2 staining. Immunohistochemical staining of PCNA was performed by the same method as described for IGF-2 staining. Anti-PCNA antibody, PC-10(No.M0879,Dako Co. Kyoto, Japan), was used at a final concentration of 20 μg/ml in PBS. All slides were observed microscopically at 400× magnification, and the ratio(%) of PCNA positive nuclei to all nuclei examined(about 400 cancer cells) was calculated at the invasive margin of the tumor. This ratio represented the PCNA labeling index (PCNA LI).

4) Statistical analysis. Statistical analysis was by Student's t test or chi-square test. For all statistical analyses, $p<0.05$ was considered to be

significant.

Results

1) Immunohistochemical staining of IGF-2 and IGF-1R. In IGF-2 positive sections, the cytoplasm of the cell was stained pale purplish red and many granules were observed in the cytoplasm at 400X magnification(Fig 2a). In IGF-2 negative sections, virtually none of the cells were stained in the same manner as in the IGF-2 positive sections(Fig 2b). The results of IGF-2 staining are shown in Table 2. The positive ratio at the invasive margin(70%) was higher than at the center of the tumor(47%). Although the positive ratios at the two sites of the normal liver were the same(83%), the details were quite different. That is, no strongly-positive case was observed at the distant site, while the majority of cases were stained strongly at the normal liver adjacent to the tumor. The typical staining pattern at the invasive margin is shown in Fig 3. A dark-red band was produced around the tumor by the strongly stained hepatocytes. In IGF-1R positive sections, the cytoplasm of the cell was stained(Fig4). The positive ratio of IGF-1R was 83%(Table 3).

2) In situ hybridization of IGF-2. Expression of IGF-2 mRNA was observed in both cancer cells at the invasive margin and at the

hepatocytes adjacent to the tumor. The cells which expressed IGF-2 mRNA were stained dark-brown(Fig. 5).

3) Relationship between IGF-2, IGF-1R and PCNA LI. In cells showing positive PCNA staining, the nucleus was either partially or entirely stained reddish brown(Fig 6). The PCNA LI for the liver metastase examined ranged from 13.0 to 88.0, with a mean of 56.9(95% confidence interval:48.5-65.3). The chi-square test showed a significant correlation between IGF-2 staining and IGF-1R staining($p=0.0019$). Furthermore, the PCNA LIs for the IGF-2 positive and strongly positive groups were significantly higher than that for the IGF-2 negative group($p=0.0065$ and $p<0.0001$ respectively, Fig. 7a). Similarly the PCNA LIs for the IGF-1R positive and strongly positive groups were significantly higher than that for the IGF-1R negative group ($p=0.0036$ and $p=0.0001$ respectively, Fig. 7b).

Discussion

Since Paget proposed his "seed and soil theory", many reports have supported this theory. For example, Radinsky[3] reported that hepatocyte derived TGF-α transduces the signal for cell proliferation and DNA synthesis by binding to epidermal growth factor receptors at liver

metastasis, Li Long et al[4] used mice and a highly liver-metastatic cell line to demonstrate that hepatocyte-derived IGF-1 stimulates the growth of liver metastasis, and HSU et al[20] reported that Platelet-derived growth factor-B (PDGF-B) increases colon cancer cell growth in vivo through a paracrine mechanism. As for IGF-2, recent reports have suggested that IGF-2 acts as an autocrine growth factor by binding to IGF-1 receptors in several human colorectal cancer cell lines[15-17] and a previous report of ours mentioned the possibility that IGF-2 was one of the autocrine growth factors in primary colorectal cancer[5]. It is well-known that hepatocytes can produce IGF-2. Nevertheless, the role of IGF-2 is not yet clearly understood. Taking the above-mentioned findings into consideration, we hypothesized that hepatocyte-derived IGF-2 stimulates cancer cell growth at liver metastasis from colorectal cancer by means of a paracrine mechanism. It is difficult, however, to prove the existence of an autocrine or a paracrine mechanism in cancer patients. Although some researchers have suggested that the expression of growth factors as demonstrated by both immunohistochemical staining and in situ hybridization is evidence of an autocrine and a paracrine activity[21], these methods cannot prove whether growth factors activate or inhibit the proliferative activity of cells. IGF-2 has been

shown to bind to the IGF-1 receptor and to activate tyrosine kinase, so that the signal for cell proliferation and DNA synthesis is transduced to the nucleus[22]. We speculated that IGF-2 might effect on increase in cell proliferation by binding to the IGF-1 receptor in cancer cells. In this study, we therefore examined the expression of IGF-2 and IGF-1R. In addition, PCNA LI was determined to serve as an indicator of the proliferative activity of cells in order to identify the effect of IGF-2. In our investigation, hepatocytes adjacent to the tumor were stained strongly by IGF-2 staining but hepatocytes distant from the tumor were not stained strongly. This suggests that hepatocytes may produce IGF-2 by the effect of liver metastasis. In addition, IGF-1R was strongly expressed at the invasive margin of the tumor and the PCNA LIs for the IGF-2 positive group were significantly higher than those for the IGF-2 negative group. These observations suggest that hepatocyte-derived IGF-2 may induce an increase in the proliferative activity of cancer cells.

In conclusion, our results can be considered indirect evidence that IGF-2 acts as a paracrine growth factor and plays an important role in tumor progression in liver metastasis from colorectal cancer. Li long et al[23] reported that IGF-1R may play a critical role in generating liver

metastasis, by presenting the fact that a cell line, which can produce liver metastasis in mouse experiment, lost the metastatic ability by merely blocking the expression of IGF-1R. Liver metastasis is a serious factor that affects patients survival. However, effective therapies for liver metastasis have not been established except for a few cases which can be curatively resected. New and effective therapies for liver metastasis must be developed as soon as possible, and IGFs and IGF-1R may well be important targets for such new cancer therapies. However, the functions of IGF-2 and IGF-1R in the human body remain poorly understood, and further study is needed to make clinical application possible.

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Figure Legends

Fig 1. A schematic cross-section of liver metastasis and normal liver tissue. IGF-2 stained sections were observed at four locations; 1) the center of the tumor, 2) the invasive margin of the tumor, 3) the normal liver adjacent to the tumor and 4) the normal liver at least 1cm distant from tumor. IGF-1R stained and PCNA stained sections, as well as in situ hybridization of IGF-2 were observed only at the invasive margin of the tumor.

Fig 2. Immunohistochemical staining with anti-IGF-2 antibody for liver metastasis from colorectal cancer. a: positive case ×400, b: negative case ×400.

Fig 3. Typical pattern at the invasive margin of IGF-2 staining(×100). A dark-red band was produced around the tumor by the strongly stained hepatocytes.

Fig 4. Immunohistochemical staining with anti-IGF-1R antibody for liver metastasis from colorectal cancer. In IGF-1R positive sections, the

cytoplasm of the cell was stained(×100).

Fig 5. In situ hybridization by anti-sense IGF-2 biotinylated labeled probe. Expression of mRNA was observed as an intense dark-brown signal(×100).

Fig 6. Immunohistochemical staining for liver metastasis from colorectal cancer with anti-PCNA antibody(×400).

Fig 7. The PCNA LIs classified by IGF-2 and IGF-1R staining. Each box represents 75% of the cases included, the area between the bars outside of the box represents 95% of the cases and the bar inside the box shows the mean value for the group. a; The PCNA LIs for the IGF-2 positive and strongly positive groups were significantly higher than those for the IGF-2 negative group. b; The PCNA LIs for the IGF-1R positive and strongly positive groups were significantly higher than those for the IGF-1R negative group.

TABLE 1 Patient Characteristics (N=30)

Variables	Age, number of cases
Average age (yrs)	62.6 (range, 45-78)
Sex	
Male	16 (53%)
Female	14 (47%)
Site of origin	
Cecum	2 (7%)
Ascending	3 (10%)
Transverse	0 (0%)
Descending	3 (10%)
Sigmoid	5 (17%)
Rectum	17 (57%)
Metastasis	
Synchronous	16 (53%)
Metachronous	14 (47%)

TABLE 2 IGF-2 Immunostaining Results in Hepatic Metastases and Host Liver Tissue from Thirty Subjects with Colorectal Cancer

Results of IGF-2 Immunostaining	Location within Host Liver Tissue		Location within Metastatic Tumor	
	Adjacent to Tumor N(%)	Distant from Tumor N(%)	At Tumor Margin N(%)	At Center of Tumor N(%)
Strong Positive	22(73%)	0(0%)	11(37%)	5(17%)
Weak Positive	3(10%)	25(83%)	10(33%)	9(30%)
Negative	5(17%)	5(17%)	9(30%)	16(53%)

TABLE 3 Results of IGF-1R Immunostaining at the Tumor Margin

	number of cases
Negative	5(17%)
Positive	25(83%)
Weak	10 (33%)
Strong	15 (50%)

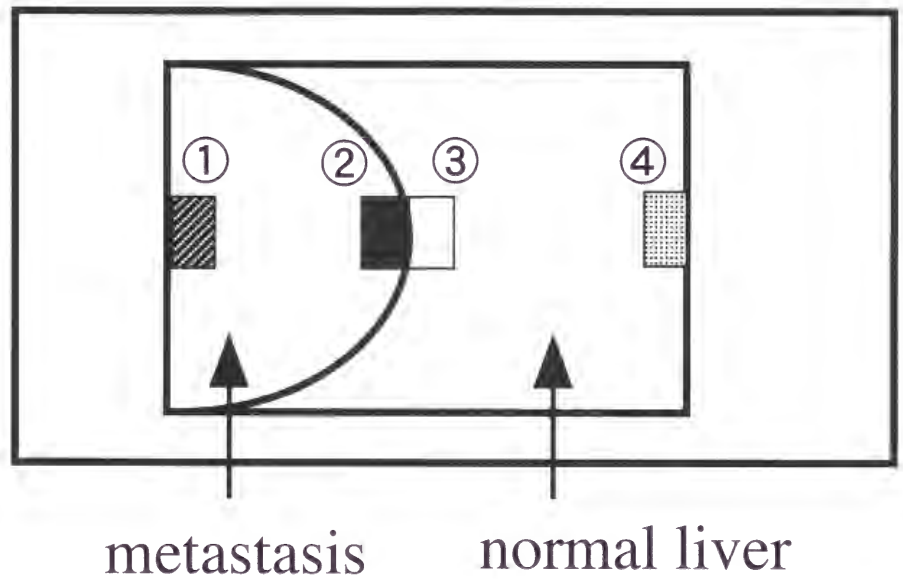


Fig.1

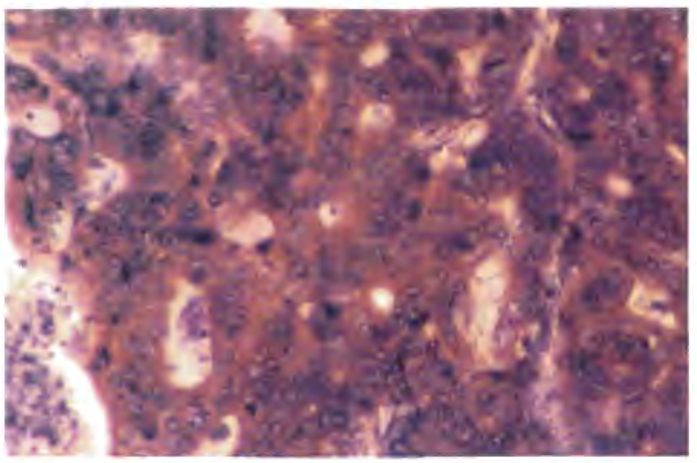


Fig.2a

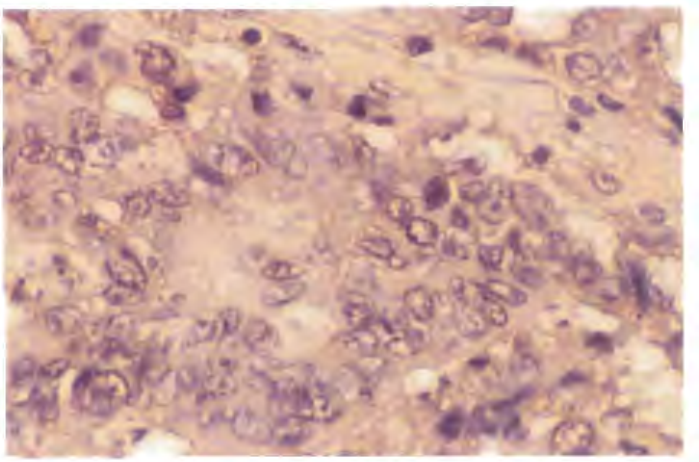


Fig.2b

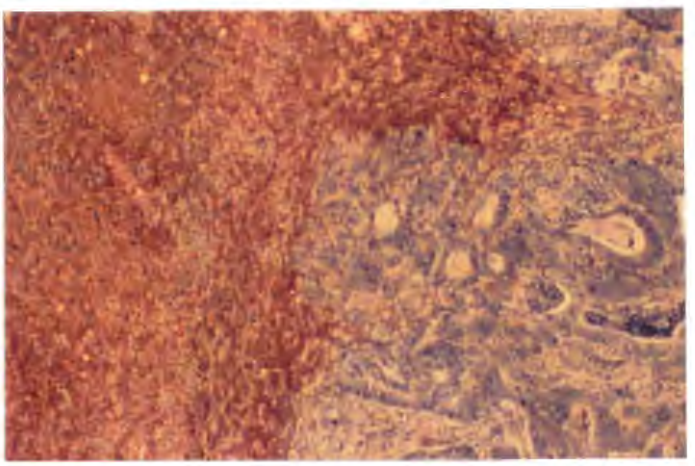


Fig.3

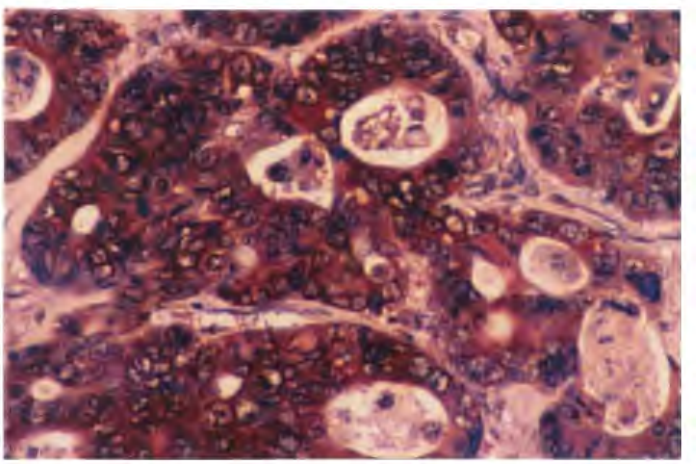


Fig.4

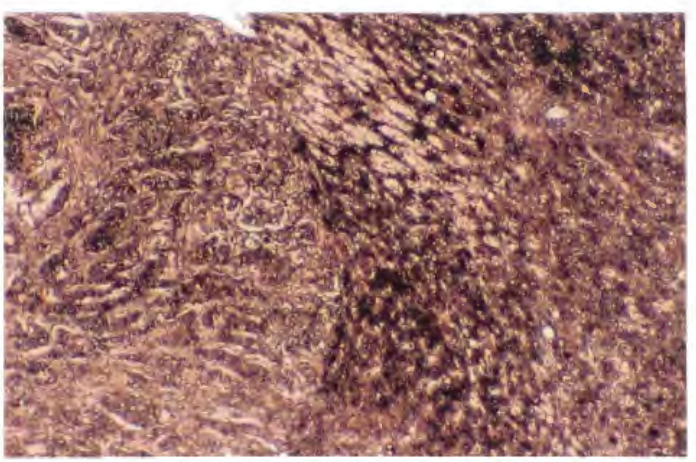


Fig.5

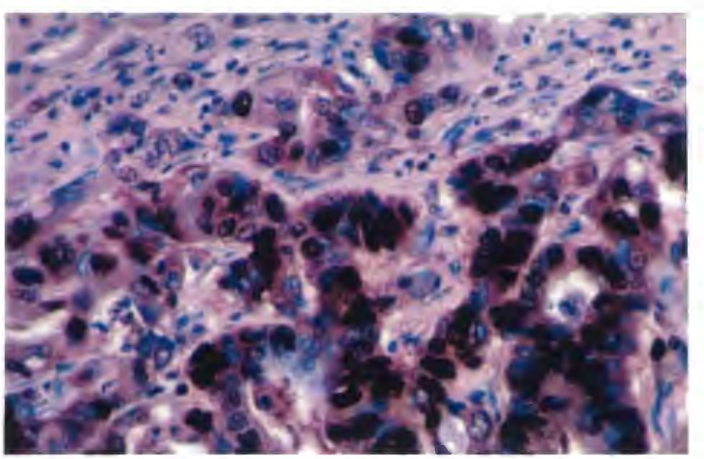


Fig.6

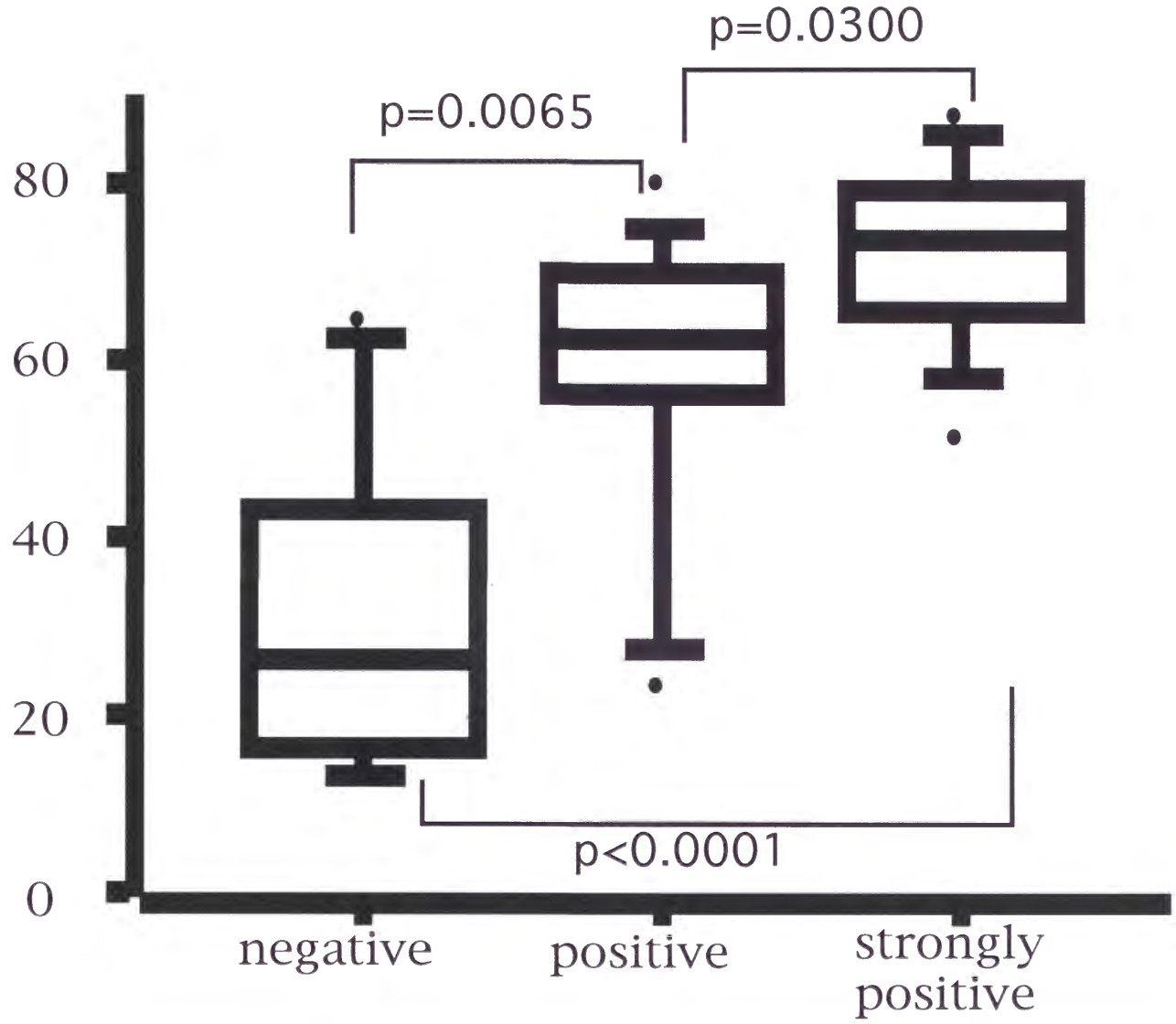
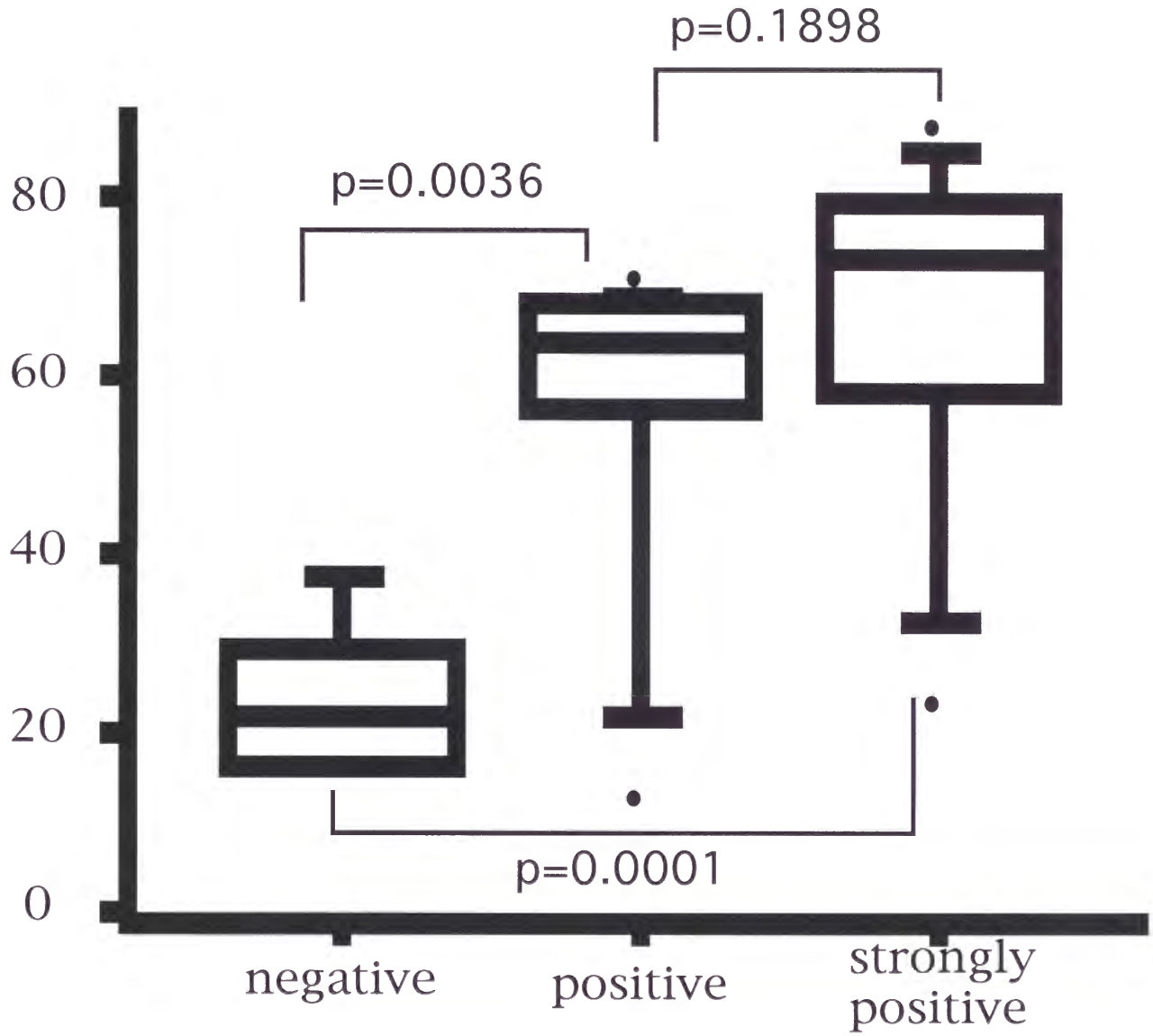

p=0.0300
p=0.0065
80
60
40
20
0
p<0.0001
negative
positive
strongly positive
IGF-2 staining

Fig.7a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80
60
40
20
0
p=0.0001
negative
positive
strongly positive
IGF-1R staining

Fig.7b